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RESEARCH ARTICLE

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# Effects of dietary β-1,3-glucan and host gut-derived probiotic bacteria on hemato-immunological indices and gut microbiota of juvenile rainbow trout (*Oncorhynchus mykiss*)

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## ABSTRACT

The effects of indigenous probiotics *Lactobacillus plantarum* and *Lactobacillus pentosus* alone, and in combination with β-1,3-glucan in juvenile rainbow trout (*Oncorhynchus mykiss*) were investigated. Eight groups were defined: control (G1), 1% β-1,3-glucan (G2), *L. plantarum* (G3), *L. pentosus* (G4), *L. plantarum* + *L. pentosus* (G5), *L. plantarum* with 1% β-1,3-glucan (G6), *L. pentosus* with 1% β-1,3-glucan (G7) and *L. plantarum* + *L. pentosus* with 1% β-1,3-glucan (G8). After eight weeks, the innate immune responses were elevated in all treated groups; however, synergistic effects were observed for anti-trypsin, bactericidal activity and respiratory burst activity in groups 7 and 8. Although the other immune responses were higher in treated groups, they did not make statistically significant differences. Checking microbiota showed that β-1,3-glucan improved conditions of indigenous probiotics. The diet 8 caused significant alterations in the intestinal microbiota by significantly decreasing the proportion of total count bacteria to lactic acid bacteria, which were demonstrated by reducing the total number of bacteria in Group 8 compared to the control group.

## Keywords

*Oncorhynchus mykiss, host gut-derived probiotics, immune response, synbiotic, β-1,3-glucan, intestinal microbiota*

## Abbreviations

RBC: Red Blood Cell
WBC:White Blood Cell
Hb: Hemoglobin
NBT: Nitro Blue Tetrazolium



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## Introduction

In recent years, cold-water fish culture has developed into a very thriving industry, due to its priority in various aspects, including its meat. This development has led to overcrowding of trout farming, combined with the increase in intensive production strategies at higher densities. Different sources of stress such as high stocking densities and manipulations, adversely affect the immune system, leading to the emergence of diseases that cause significant economic losses and prevent the sustainable development of the aquaculture industry [1]. Thus, developing a safe and viable alternative of veterinary chemicals/antibiotics in aquaculture health management has received much attention [2]. The use of conventional chemotherapeutics including antibiotics and chemical disinfectants, not only pollutes the water, but also gradually causes the occurrence of resistant bacteria [3], and contributes to the accumulation of drug residues and reduces consumer preference for aquaculture products [4]. As a result, various alternative strategies for the antibiotic use have been proposed, among them, the use of pre- and pro-biotics having better efficacy. Probiotics are defined as live microbial feed supplements, and used as environment-friendly treatments to control diseases [5]. Previous research efforts have demonstrated that probiotics can improve disease resistance and immune responses of aquatic animals [6,7]. The most common probiotics used in aquaculture are *Lactobacillus spp.*, *Bacillus spp.*, *Vibrio spp.*, *Saccharomyces spp.* and *Enterococcus spp.* [8]. Moreover, the composition of an intestinal microbiota held by animals is often limited in several autochthonous species [9]. It has been suggested that the fish intestinal autochthonous bacteria might be a vital source of potential probiotics and some autochthonous bacteria might be treated as likely probiotics [10]. Studying the interaction between the gut autochthonous bacteria and the host immune system may reveal some beneficial or harmful effects exerted by these presumptive probiotics. Therefore, the investigation of the immunologic functions of autochthonous gut bacteria is relatively less [11]. The combined use of two or more probiotics and growth-promoting additives are new concepts in aquaculture [12]. Until now, experiments typically tested the effects of only one probiotic [13]. However, a few studies have combined different stimulants to amplify the impacts of cultured aquatic animals.

Evidence of the beneficial effects of probiotics gave birth to the concept of prebiotics [14], which are defined as indigestible (by the host) feed components that provide beneficial impacts to the host through their selective metabolism by favorable bacteria in the gastrointestinal tract (GI) [15]. β-glucans are prebiotics commonly used in fish and are naturally occurring polysaccharides found in the cell walls of the yeast *Saccharomyces cerevisiae*. Other sources, such as brewers' yeast, torula yeast *Candida utilis*, fungi, and algae, are also currently used [16]. A Previous study indicated that the use of prebiotics containing β-glucan and MOS was found to improve the immune system performance of common carp *Cyprinus carpio,* beluga *Huso huso*, and sea cucumbers *Apostichopus japonicus* [13,17,18].

Synbiotics, contain probiotics and prebiotics, have been introduced, and first used to enhance the immune responses of fish since 2005 [19]. In aquatic animals, it seems common to manipulate the gastrointestinal microbiota using synbiotics (probiotic with prebiotic) that alter the conditions of the gastrointestinal tract in favor of certain bacterial species. Mainly due to the synergistic effects of synbiotics, it may increase growth efficiency in endogenous populations, improve survival and increase nutritional supplementation for the living microbial population in the host gastrointestinal tract and reduce susceptibility to disease.

Although the use of single pre- and pro-biotics are now widely accepted in aquaculture, only a few studies have focused on the effects of synbiotics in farmed aquatic species, indicating that they might yield better results than the individual pre-and pro-biotics. This study was carried out to evaluate the effects of single or combined supplementation of two endogenous probiotics, along with the prebiotic (β-glucan). In this study we also aimed for the selection of a combination of prebiotic and probiotic to establish a suitable synbiotic formulation based on their effect on the intestinal flora and immunity in the rainbow trout. The candidate probiotics, two strains of *Lactobacillus plantarum* and *Lactobacillus pentosus,* were isolated from the gut of Tor grypus in our previous work [20,21].

## Results

### *Bacteriological examinations and identification*

Hematological indices were affected significantly in probiotic-treated experimental groups. Probiotic-fed groups showed a significant increase in RBC compared with the control group on day 60 ($p < 0.05$) (Table 1). The highest increase was observed in G5 and G8 groups, respectively.

Probiotic-fed groups had a significant difference in WBC count compared with the control group on day 60 ($p < 0.05$), and the highest WBC counts were observed in the G6 and G8 groups, respectively. The lowest WBC count was observed in G3 group after 60 days (Table 1).

Probiotic-fed groups had significantly different levels of Hb than that in the control group on day 60 ($p < 0.05$), and the highest Hb levels were observed in G8, G4 and G7 groups on day 60, respectively (Table 1).

### *Nonspecific immunity parameters*

Total Ig was significantly affected by diet in G6, and G7 compared to the other treatments ($p < 0.05$, Fig. 1). Moreover, a synergistic effect between *L. pentosus* and *L. plantarum* with β-glucan was observed. Anti-trypsin was higher in fish receiving dietary *L. pentoseus* with *L. plantarum* (G5) and *L. plantarum* in combination with β-glucan (G6), compared to fish fed the control diet and in other groups ($p > 0.05$, Fig. 2).

Among the non-specific humoral immune parameters, alternative complement activity was significantly boosted by a synergistic effect of β-glucan and the *L. pentosus* (G7) than dietary separately. Alternative complement activity was significantly higher in the serum of G 7 than that in the other treatments (Fig. 3).

The serum lysozyme activity increased marginally in all the treated groups at different times of trial feeding with some exceptions (Fig. 4). At day 60, G5 and G8 had higher serum lysozyme activity than other groups ($p > 0.05$).

After feeding the pro- and pre-biotic for 60 days, the ability to kill *L. garvieae* was more exceptional in the serum of G4, G5 and G8 than the control and other treatments ($p > 0.05$) (Fig. 5).

After sixty days of post-feeding (dpf), the G4, G5,

**Table 1**
Blood parameters in *O. mykiss* fed with different pro-, pre- and syn-biotic diets for 60 days.

| Blood parameters | Groups | Time(0) | Time(60) |
|---|---|---|---|
| RBC ($10^6/\mu L^{-1}$) | Control | 2.88 ± 0.38 | 3.43 ± 0.45$^{ab}$ |
| | G2 | 3.15 ± 0.32 | 2.31 ± 0.89$^{b}$ |
| | G3 | 3.68 ± 0.36 | 3.23 ± 1.46$^{ab}$ |
| | G4 | 3.35 ± 0.32 | 2.89 ± 0.94$^{ab}$ |
| | G5 | 3.17 ± 0.36 | 3.74 ± 0.83$^{a}$ |
| | G6 | 3.77 ± 0.45 | 2.46 ± 1.16$^{ab}$ |
| | G7 | 3.44 ± 0.27 | 3.04 ± 0.58$^{ab}$ |
| | G8 | 2.97 ± 0.36 | 3.66 ± 0.55$^{a}$ |
| WBC ($10^3/\mu L^{-1}$) | Control | 13.33 ± 1.61 | 13.50 ± 1.81$^{b}$ |
| | G2 | 12.2 ± 3.20 | 15.25 ± 2.62$^{ab}$ |
| | G3 | 11.33 ± 1.61 | 13.35 ± 8.81$^{b}$ |
| | G4 | 15.2 ± 3.20 | 19 ± 5.17$^{ab}$ |
| | G5 | 12.625 ± 3.95 | 15.9 ± 6.82$^{ab}$ |
| | G6 | 15.1 ± 3.53 | 21 ± 5.11$^{a}$ |
| | G7 | 17.1 ± 2.03 | 19.5 ± 6.51$^{ab}$ |
| | G8 | 12.25 ± 3.75 | 22.5 ± 7.5$^{a}$ |
| Hb (g/dl) | Control | 9.11 ± 1.09 | 11.63 ± 0.98$^{ab}$ |
| | G2 | 10.51 ± 1.09 | 11.64 ± 0.36$^{ab}$ |
| | G3 | 11.65 ± 1.13 | 10.90 ± 0.81$^{b}$ |
| | G4 | 11.07 ± 1.01 | 12.46 ± 1.05$^{a}$ |
| | G5 | 10.37 ± 1.01 | 11.44 ± 1.04$^{ab}$ |
| | G6 | 11.55 ± 1.36 | 11.42 ± 0.53$^{ab}$ |
| | G7 | 11.21 ± 1.09 | 12.22 ± 0.46$^{ab}$ |
| | G8 | 9.22 ± 1.02 | 12.48 ± 0.65$^{a}$ |

*Each value represent as a mean ± standard error (n = 9). Different lowercase superscripts denote significant differences within columns ($P < 0.05$).

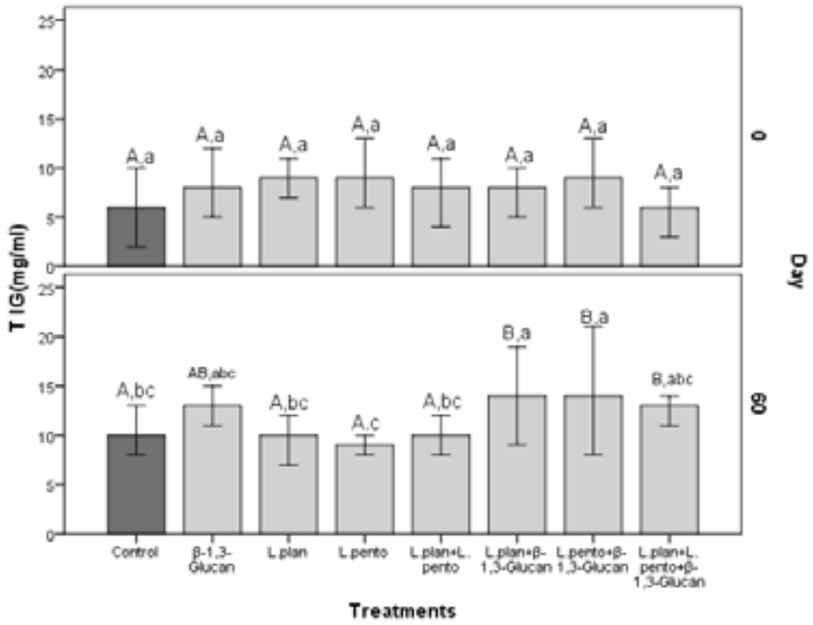


**Figure 1**
Total Ig of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

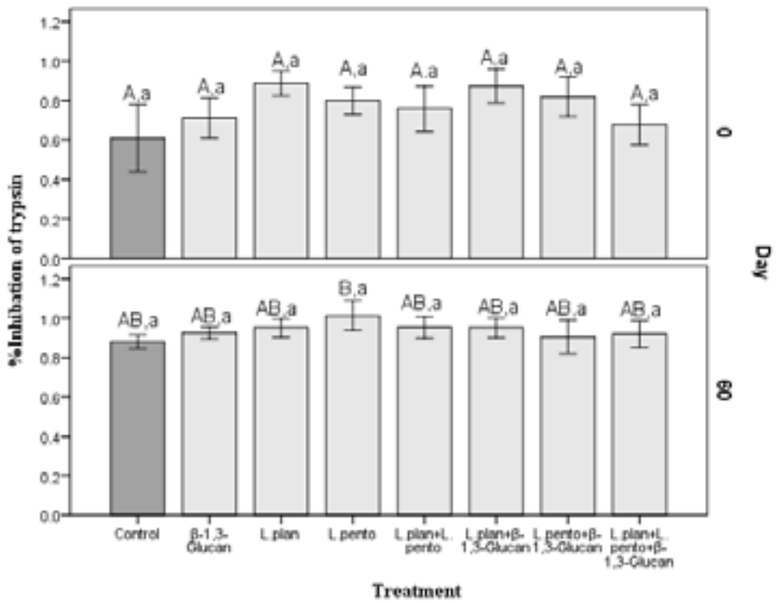


**Figure 2**
Anti-trypsin activity of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).





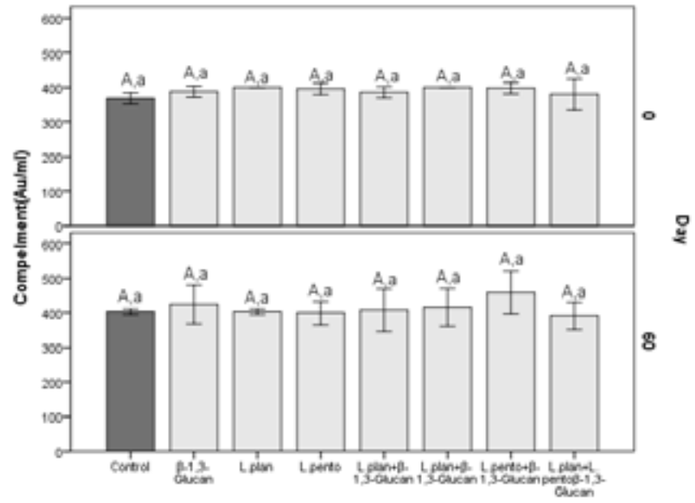


**Figure 3**
Serum complement activity of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

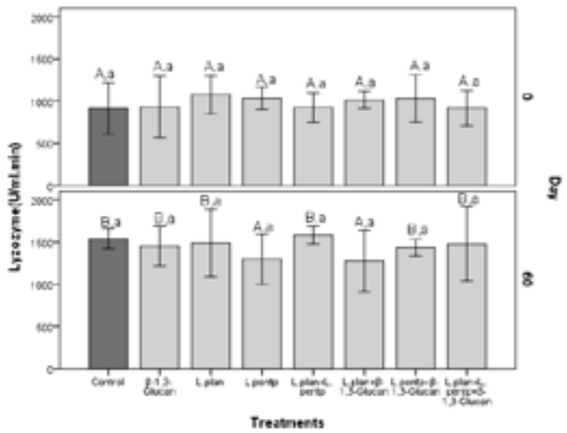


**Figure 4**
Serum lysozyme activity (unit/ml$^{-1}$) of O. *mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

and G8 showed higher oxide anion production upon stimulation with pro- and pre-biotics compared with the control group (Fig. 6).

Serum myeloperoxidase content was positively affected by both *L. pentosus* alone and *L. pentosus* with *L. plantarum* and β-glucan diets (Fig. 7). It was higher in fish fed in G3, G5 and G7 groups than those in the control group ($p > 0.05$).

Serum Anti-trypsin content was positively affected by both *L. plantarum* alone and *L. pentoseus* with *L. plantarum* and *L. plantarum* with β-glucan diets (Fig. 8). It was higher in fish fed with G3, G5, and G6 diets than fish fed with the control diet ($p > 0.05$).

### *Microbiological assay*

Before the pro- and pre-biotic feeding, the fish, showed a low detectable LAB level in the entire intestines. Although in G1 an increase in viable counts at day 60 was seen, the only viable count of LABs significantly increased in a time-dependent manner in the intestine of G8 (Table 2). Content of the intestinal bacteria counts in fish fed with pro- and pre-biotics showed significant differences compared with the control ($p < 0.05$).

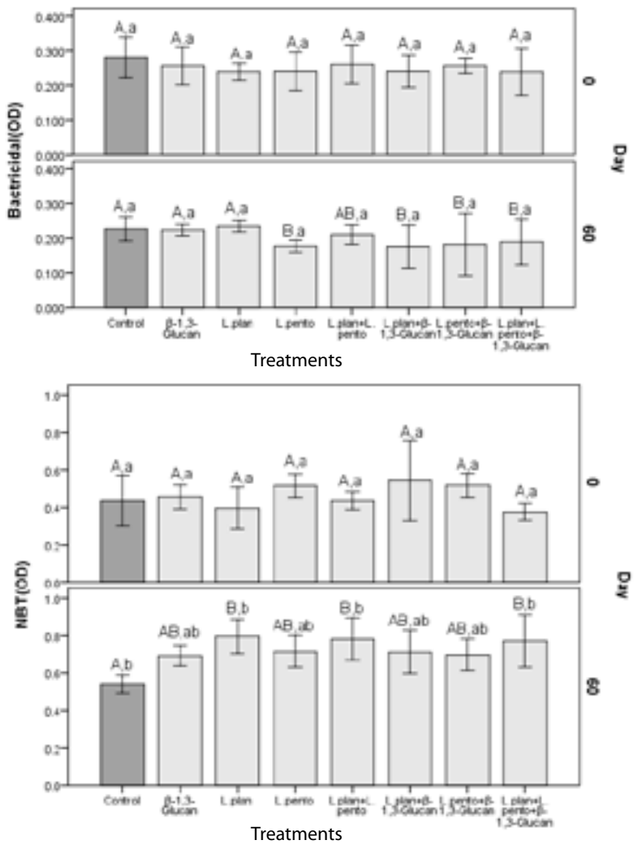


**Figure 5**
Serum bactericidal activity of *O. mykiss* in eight experimental groups . Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).




**Figure 6**
Reduction in NBT of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p$ <0.05).

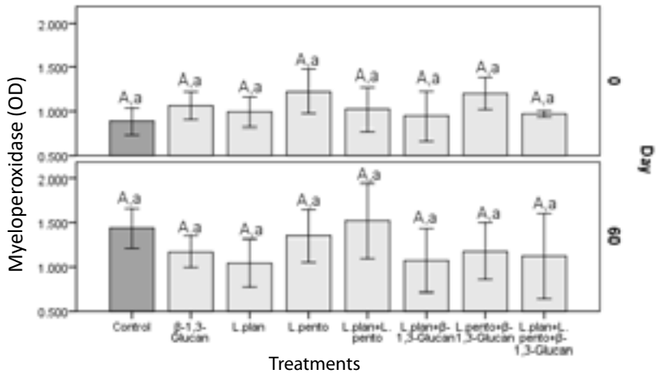


**Figure 7**
Serum myeloperoxidase activity of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letters express significant differences among different treatments ($p < 0.05$).

## Discussion

This study was performed to assess the effects of two indigenous bacteria *L. plantarum* and *L. pentosus* separately and combined with β-1,3-glucan (a yeast cell wall-derived immune stimulant) on the general innate immune system and microbial flora in rainbow trout (*O. mykiss*). The results demonstrated that continuous feeding of symbiotic (pre- and pro-biotic) for 60 days led to increased several immune accelerated parameters of rainbow trout.

β-1,3-glucan alone cannot be hydrolysed by pancreatic or brush border digestive enzymes in the proximal intestinal tract of human and fish. β-1,3-glucan has repeatedly been shown to stimulate innate immune functions in various fish species [22, 23], and the expected activities were confirmed in *O. mykiss*. In this study, immune parameters examined, including lysozyme, and alternative complement pathway (ACP) activity, respiratory burst, bactericidal activity, and total Ig in serum, were stimulated by β-1,3glucan more than the control group. Overall, these results confirm that β-1,3-glucan can be an efficient stimulant of innate immunity in *O. mykiss*, as demonstrated previously in other studies [24]. Improvement of the immune system in fish fed with a β-1,3-glucan diet might be attributed to fermentation in the large intestine or colon by lactic acid-producing bacteria (LAB), enhancing their relative populations, elevated health status and increased colonization of the LAB compared to the control diets.





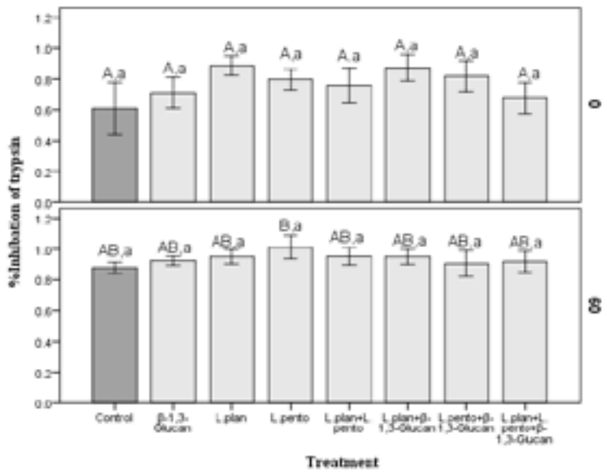


**Figure 8**
Anti-trypsin activity of *O. mykiss* in eight experimental groups. Data represent mean ± SD of a triplicate set of nine fish. Different capital alphabetic letters on each bar indicate significant differences at different sampling time, and different lower letter letters express significant differences among different treatments ($p$ <0.05).

**Table 2**
Total viable counts and total lactic acid bacteria (LAB) from the digestive tract of *Tor grypus*. MRS: de Man, Rogosa and Sharpe. Values are presented as mean ± SD (n = 9). Different small alphabetic letters in the same column show significant differences ($p < 0.05$).

| Groups | Groups | Time (0) | Time (60) |
|---|---|---|---|
| Total counts $10^5$ cfu $mL^{-1}$ | Control | 34.33 ± 2.08 | 27 ± 8.50$^{ab}$ |
| | G2 | 28.2 ± 6.53 | 12.83 ± 3.31$^{c}$ |
| | G3 | 23.25 ± 7.13 | 29.16 ± 5.81$^{ab}$ |
| | G4 | 26.2 ± 6.53 | 38.5 ± 7.14$^{a}$ |
| | G5 | 28.2 ± 6.53 | 24.33 ± 6.88$^{bc}$ |
| | G6 | 23.25 ± 7.13 | 26.83 ± 9.74$^{ab}$ |
| | G7 | 26.2 ± 6.53 | 21.83 ± 5.45$^{bc}$ |
| | G8 | 27.66 ± 7.76 | 19.33 ± 4.36$^{bc}$ |
| MRS counts (Lactobacillus) $10^2$cfu $mL^{-1}$ | Control | 1/2 ± 0/44 | 1/2 ± 0/54$^{e}$ |
| | G2 | 1/5 ± 0/7 | 5/2 ± 1/48$^{de}$ |
| | G3 | 1/33 ± 0/57 | 23/66 ± 5/08$^{b}$ |
| | G4 | 1 ± 0 | 9/83 ± 2/48$^{cd}$ |
| | G5 | 1/25 ± 0.5 | 12/16 ± 4/66$^{cd}$ |
| | G6 | 1 ± 0 | 7/5 ± 2/88$^{cde}$ |
| | G7 | 1/25 ± 0/5 | 13 ± 1/54$^{c}$ |
| | G8 | 1 ± 0 | 33/66 ± 6/08$^{a}$ |

Regarding hematological parameters of rainbow trout in experimental groups, the *Lactobacillus* probiotics (*L. plantarum* and *L. pentusus*) not only act as immune promoter, but also cause higher RBC, WBC, hematocrit and hemoglobin (Table 1). Blood parameters are essential tools for assessment of the physiological stress response and general health conditions of fish during nutritional and environmental changes [25]. In the current study, G8 treatment significantly affected RBC of rainbow trout. Similarly, Rodriguez-Estrada et al. [26] reported that hematological indices were enhanced by the supplementation of inactivated *Enterococcus faecalis* in rainbow trout diets. The higher RBC count in the blood of fish fed with diets supplemented with $1\times10^8$ CFU/$g^{-1}$ of *Lactobacillus* indicated stimulation of fish defensive mechanisms against pathogens, positive effect on fish health and the improvement of the immune functions of blood [27, 28]. The increase in the number of WBC in fish fed with probiotics may indicate stimulation of the innate immune system, possibly resulting in the improvement of the defensive systems against environmental stress or pathogens, because WBCs are regarded as the first lines of defense [29]. Similarly, significant enhancements of total leukocytes number in *Oreochromis niloticus* [30] and *Oncorhynchus mykiss* [31] fed with probiotic supplemented diets have been reported. According to Silva et al. [32] and Falcon et al. [33], the reduction in the total number of circulating cells implies downfall of immune resistance predisposing animals to the pathogenic infection. This indicates that the fish that were fed the β-1,3-glucan diet (G2) and $1\times10^8$ CFU/$g^{-1}$ of *L. plantarum* (G3) are more susceptible to possible disease outbreaks than those fed with the other doses (Table 1). Moreover, the reduction in hematocrit percentage in control group (Table 1) may indicate that they are more vulnerable to stress arising from the experimental management or the pathogenic load naturally present in the culture environment [34]. This is also indicated by other blood variables analyzed in this study. The hemoglobin content in the blood plays a vital role and serves as oxygen transport element to the body tissues. However, it should be noted that the *L. plantarum*, in combination with β-glucan, had the highest levels of hemoglobin. The increase in its contents indicates a greater supply of oxygen to the fish and consequently, improves the welfare of fish [27]. This demonstrates that *Lactobacillus* supplementation increases the availability of oxygen in fish blood, resulting in beneficial health effects.

The results of the present study showed that fish fed diets supplemented by *L. plantarum* and *L. pentoseus* along with β-glucan showed higher serum lysozyme activity than the other groups ($p < 0.05$). Accordingly, the more elevated WBC measured was concomitant of increased serum lysozyme activity in all treatments. The use of probiotic and synbiotic has been carried out in Nile tilapia (*Oreochromis niloticus*) [35,36,37], in rainbow trout (*Oncorhynchus mykiss*) [38], rockfish (*Sebastes schlegeli*) [39], in Atlantic salmon (*Salmo salar*) [40], in the yellow croaker *Larimichthys crocea* [41], and in the Japanese flounder *P. olivaceus* [42] *Epinephelus coioides* [43], and *Cyprinus carpio* [44].

The presence of protective proteins in fish blood such as complements, acute phase proteins, lysozyme, transferrin and anti-proteases can be evaluated by serum bactericidal activity, which are considered non-specific responses to inhibit the growth of infectious microorganisms [45]. Results of the current study revealed that serum bactericidal activity elevated in fish which were fed with probiotic (G4 and G5) and synbiotic enriched diets (G8) compared with the control group and G2 (β-1,3-glucan) similar to previous reports in other fish species fed with probiotics [35,46]. Improvement of the immune system in fish fed a synbiotic diet and indigenous probiotic, which might be attributed to elevated health status, increased colonization of the probiotic compared with the control diets. Moreover, fish fed synbiotic diet had the highest serum bactericidal activity, which was associated with the highest serum lysozyme and complement activity in these groups. It has been suggested that synbiotics can induce immune system by short-chain fatty acids, which can partially act as a source of energy for intestinal epithelial cells and also may have a role as a messenger between gut microbiota and immune system by modulation of signaling and transcriptional pathways [47]. In the absence of specific opsonization, alternative immune responses could depend on the presence of mannose receptors and toll-like receptors (TLRs) in microbes, which bind to mannose and glucans, leading to enhanced phagocytic and bactericidal abilities in phagocytes and neutrophils (48,49). Mannan oligosaccharides bind with and block receptors on pathogens, preventing their colonization or invasion of the host. MOS also enhances the liver's secretion of material rich in mannose-binding lectin, which binds the bacterial capsule and triggers the complement cascade [50].

The alternative complement pathway activity (ACP) can be measured through the determination of serum hemolytic activity in response to foreign red blood cells [23]. We found that fish fed diet supplemented with β-1,3-glucan and *L. pentosus* (G7) had higher serum hemolytic activity than fish in control and the group fed with *Lactobacilus plantarum* + *Lactobasilus pentoseus* with 1% β-glocun (G8). Similarly, Van Doan et al. [36] reported that inclusion of 10 g/$kg^{-1}$ LMWSA in the diet significantly improved serum ACP in Nile tilapia compared with fish fed 20 and 30 g/$kg^{-1}$ LMWSA. These results were also in accordance with the previous results in different species fed diet supplemented with sodium alginate [41,51]. On the other hand, the finding of the current study showed that fish fed the diet supplemented with *L. pentosus* had the lowest serum haemolytic activity in comparison with other experimental groups as also in green terror (*Aequidens rivulatus*) [52]. In line with the results of this study, it has been reported that PA in combination with GOS significantly increased ACP in the rainbow trout [53] and rockfish [39] compared with those in the control pro- and pre- fed fish.

Supplementing diet with synbiotics (β-1,3-glucan /*L. pentosus* and β-1,3-glucan *L. plantarum*) in the rainbow trout affected serum globulin level. Present results concur with the reports of previous researchers that using immunostimulants increased total serum protein, albumin, globulin, and immunoglobulins in different fish species [54,55,56].

This study demonstrated that the combined use of β-1,3-glucan with *L. pentosus* and *L. plantarum*, positively affected cellular immunity as well as humoral immunity of juvenile rainbow trout. The effect measured in the synbiotic-fed group with the exception of NBT, anti-trypsin and bactericidal activity, was higher than that predicted by the individual effects measured in the prebiotic and probiotic-fed group. This study represents the synergistic effects of *Lactobacillus plantarum* and *Lactobacillus pentoseus* with β-1,3-glucan.

The intestinal microbiota of fish plays a key role in nutritional function, enhances growth performance, and stimulates the host immune system and resistance against pathogens [53]. Singular or combined administration of β-1,3-glucan and probiotic *L. pentosus* and *L. plantarum* were showed an interesting and remarkable result in the number of lactic acid bacteria in the rainbow trout intestine. The *Lactobacillus plantarum* + *Lactobacillus pentosus* with 1% β-glucan (G8) had a significantly increased LAB in the experimental groups. But the interesting point was that the *Lactobacillus plantarum* group (G3), when used alone, had a better performance than combined administration of β-1,3-glucan (G6) in the number of LAB. In conclusion, the ability of *Lactobacillus plantarum* to colonize and modify the intestinal microbiota as a potential probiotic strain, was confirmed. Dietary fermentable β-1,3-glucan are components that are not hydrolyzed by digestive enzymes of non-ruminant animals such as fish and consequently are the main substrates for bacterial fermentation in the gut [57]. Therefore, an increase in gut microflora of G8 may have occured [58]. In our study, the higher degree of adhesion of specific microbes that are supplemented through diets may be the reason for enhanced innate immune responses and hematological indices of fish.

In conclusion, the diet administration of *L. plantarum* and *L. pentosus* that showed to colonize and modify the intestinal microbiota as a potential and host derived probiotic strain, was confirmed. The selected probiotic strains isolated from fish are safe and capable of surviving and colonizing the fish intestinal mucus, as well as antagonizing the resident microbiota. The results of the present study strongly suggest that the dietary combination of *L. plantarum*, *L. pentosus* and β-1,3-glucan is significantly effective to stimulate some hematological factors of *O. mykiss*, compared with their singular administration. The *Lactobacillus plantarum* + *Lactobacillus pentosus* with 1% β-1,3-glucan (G8) caused significant alterations in the intestinal microbiota by significantly decreasing the bacterial diversity, demonstrated by reducing the total number of bacteria than the control group and boosted immune responses of rainbow trout. Therefore, they could be considered as a useful alternative to chemotherapeutic treatments to promote fish health status. Future researches are needed to be carried out under a more holistic approach, in which different rearing conditions (i.e., stocking densities, water temperatures, oxygen levels and exposure to pathogens) and concomitant use of antibiotics or vaccines and indigenous probiotics are used. In addition, the study of the colonization of these bacteria in the digestive

**Table 3**
primers sequences that were used for identification of different lactoacid bacteria

| primer | 5'Sequence3' | Specificity |
|---|---|---|
| forward | GCCGCCTAAGGTGGGACAGAT | *L. plantarum* |
| Revers | TTACCTAACGGTAAATGCGA | |
| | CGCCGCCCGGGTGAAGGTG | |
| Forward | CTGCTGGGACGAAAAG | *Lb pentoseus* |
| Revers | CTGCTGGGACCTTAA | |

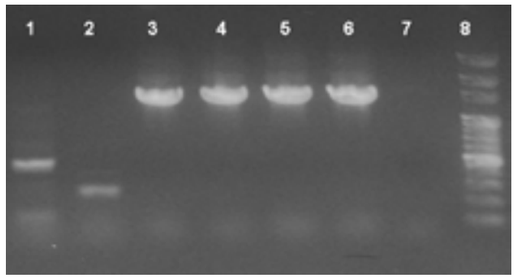


**Figure 9**
PCR amplification of 16 SrRNA genes for the identification of lactobacilli species. 1: PCR products of isolate 1 suspected of Lactobacillus pentosus with species-specific primers (465 bp), 2: PCR products of isolate 2 suspected of Lactobacillus plantarum with species-specific primers (284 bp), 3, 4, 5 and 6: PCR products on DNA isolated from suspected bacteria with universal lactobacilli primers, 7: negative control, 8: M: molecular marker 100 bp.

system by the method of DGGE is needed in order to provide a more comprehensive analysis on the effects of combined use of these probiotics and the prebiotics.

## Material and methods

### *Isolated bacteria*

*Lactobacillus plantarum* subsp. *Plantarum* and *Lactobacillus pentoseus* were used in food supplementation. Bacterial identification was primarily performed based on colony and cell morphology, Gram staining, and biochemical testing and according to their high in vitro probiotic characteristics. The confirmation of the probiotic bacteria isolated from the intestine of *Tor grypus* was performed using PCR analysis for the ribosomal RNA (rRNA) gene, as described by Mohammadian et al. [46]. The sequences of primers used in this study are shown in Table 3. The PCR was carried out on a PC 707 thermal cycler (Termocycler, Mastercycler Gradient, Eppendorf, Germany). PCR reaction was performed in a final volume of 25 µL containing 3 µL cDNA and 22 µL PCR mixture consisting of Taq DNA polymerase (1 unit), forward and reverse primers (100 nM), 1X PCR buffer, $MgCl_2$ (2 mM), and dNTPs (100 mM). The PCR was performed after 2 min of initial denaturation at 92°C, and 35 cycles of 30 s of denaturation at 95°C, 45 s of the annealing at 57°C, 45 s of primer extension at 72°C and 5 min of final extension. Amplification products were analyzed by electrophoresis in 1% (w/v) agarose gel (Fig. 9) containing ethidium bromide (1 mg/ml$^{-1}$) [23].

### *Experimental Setup*

The experiment was conducted at the laboratory of the aquatic health of The Veterinary Faculty of Chamran University of Ahwaz, Iran. Six hundred juvenile rainbow trouts (10.4±2.4 g) were randomly divided into eight experimental groups with three replicates each following a complete block design.

Experimental diet of each group were as follows: basal diet (G1: control), basal diet supplemented with 1% β-1,3-glucan (G2), *Lactobacillus plantarum* (G3), *Lactobacillus pentoseus* (G4), *Lactobacillus plantarum* + *Lactobacillus pentoseus* (G5), *Lactobasilus plantarum* with β-1,3-glucan (G6), *Lactobacillus pentoseus* with 1% β-1,3-glucan (G7) and *Lactobacilus plantarum*+*Lactobasilus pentoseus* with 1% β-1,3-glucan (G8).

### *Rearing*

Fish were obtained from local fish farming and the healthy fish were kept and acclimatized in 1000-L water tanks for 2 weeks. Fish health status was verified by physical examination (excess of mucous secretion, normal coloration, erosion of scales or fins, skin, bulging of eyes and presence of cysts, spots or patches over the body and gills) and behavioral signs (swimming and feeding reflexes). Water quality parameters including dissolved oxygen (DO) and pH were measured daily, whereas nitrate, nitrite and ammonia concentrations were monitored bi-weekly. Nitrate, nitrite and ammonia concentrations were determined using a portable Hach DR/2400 spectrophotometer. Water temperature, DO, and pH ranged from 14.8-19.4 °C, 6.6-6.9 mg/L$^{-1}$, 6.84-7.06, respectively.

### *Microorganisms*

The potential probiotic bacteria *Lactobacillus plantarum* and *L. pentaseus* were previously isolated from the gut contents of healthy *Tor grypus* and identified as potential probiotics based on diverse *in vitro* tests [23]. De Man, Rogosa and Sharpe broth (MRS) were used to grow *L. plantarum* and *L. pentasus* strains (48 h at 25 ˚C). Bacterial density was estimated via McFarland standard tube No. 0.5, OD= 0.132 at 600 nm and correlated with colony forming unit (CFU) counts using serial dilution and spread plating on MRS agar. The bacteria were subsequently harvested by centrifugation at 1500 g for 15 min in sterile phosphate-buffered saline (PBS). All prepared diets were packed in sterile propylene containers and stored at 4°C for weekly use.

### *Sample Collection*

Blood samples were collected at day 0 and 60 of the experimental period. At the end of the trial, five apparently healthy fish (no obvious skin lesions) from each replicate tank (15 fish per treatment) were anesthetized with 400 mg/L$^{-1}$ phenoxy ethanol. The blood samples were withdrawn from the caudal vein using 1.0 ml non-heparinized syringes. A part of collected blood was transferred into heparinized microtube and kept on ice for further hematological assay and the residue was allowed to clot at room temperature (for 60 min) and subjected to centrifugation (3000 g, 10 min, 4°C) to separate serum. Serum samples were stored at 80°C for further analysis.

### *Hematological Parameters*

The blood was diluted with appropriate diluting fluids and RBC and WBC counts were determined using improved Neubauer hemocytometer [59]. Hemoglobin concentration (Hb) was measured spectrophotometrically (Jenway 6400, UK) at 540 nm by the cyanomethemoglobin method [60]. Hematocrit percentage (Hct%) was measured with the microcentrifuge method (Micro-hematocrit centrifuge, 346, UNIPAA, Poland) for 10 min in duplicate.

### *Immunological Parameters*

The effect of treatments on immune responses was evaluated by assessment of the lytic activity of lysozyme against *Micrococcus lysodeikticus* [61] and oxidation of the tetramethyl-benzidine by myeloperoxidase enzyme [62]. Nitrobluetetrazolium (NBT) reduction assay was carried out using the spectrophotometric method [46]. The lysis of the 1% rabbit red blood cells and 1% sheep red blood cells coated with rabbit anti-sheep erythrocyte based on Leiro et al. [63] were detected as the activity of the alternative and classical pathway of complement, respectively. The active and heat-inactivated serum bactericidal effects against *L. garvieae* were evaluated using the broth-microdilution method [64]; the difference between the activity of the active and inactivated serum was accounted as the antibacterial activity of each sample. The zinc sulfate precipitation method [65] was used for the measurement of the total immunoglobulin.

The humoral immune response against *L. garvieae* was detected using the micro-agglutination test [66]. Serum anti-protease activity was performed [66] by incubating 10 µl of serum with 20 µg of trypsin dissolved in 100 µl of Tris-HCl (50 mM, pH 8.2). In blank serum, 100 µl of Tris-HCl was added to 10 µl of serum, instead of trypsin in Tris-HCl, and in the positive control, no serum was added to trypsin. All tubes were made up to 200 µl with Tris-HCl and incubated for 1 hour at room temperature. After the incubation, 2 ml of 0.1 mM substrate BAPNA (Na-benzoyl-DL-arginine-p-nitroanilide HCl, Sigma chemicals), dissolved in Tris-HCl (containing 20 mM calcium chloride), was added to all tubes and incubated for further 15 minutes. At the end of incubation, the reaction was stopped by adding 500 µl of 30% acetic acid. The optical density was measured at 410 nm by using a UV-Visible spectrophotometer (Shimadzu UV-1601). The percentage trypsin inhibition was calculated from the following formula: Trypsin inhibition (%) = (A1-A2/A1) x 100, where A1 = control trypsin activity (without serum); A2 = activity of trypsin remained after addition of serum.

### *Bacterial Community Analysis*

Micro floral analysis was done as described by Mohammadian et al. [67]. Total and *Lactobacillus* counts in fish intestines were determined by plate counting on TSA and MRS agar, respectively. The intestine of the experimental fish (6 fish from each treatment) was sampled just prior to starting on the experimental diets, and 60 days past the probiotic and prebiotic feeding and to determine the effect of both combination (symbiotic effect), the supplemented diets were stopped for 24 h [67,68]. This was done by aseptically dissecting the fish after overdose (1 ml/L$^{-1}$) of anesthesia (Benzocaine; Sigma-Aldrich Co., St Louis, MO, USA) and removing a portion of posterior intestine that was finely chopped. All steps were carried out under sterile conditions. One gram of the sample was homogenized with 9 ml of sterilized phosphate buffered saline (PBS, 0.1 M, pH=7.0) and stirred for 1 min in stomacher (Orugan Stomacher, Tokyo, Japan). Subsequently, dilution series were prepared from the homogenate and plated in the MRS and TSA media. The plates were incubated at 30°C for 48 h before counting. Confirmation of the isolated bacteria from the gut was done in the previous work by using morphological, biochemical, and molecular tests adopted from Bergey's manual of systematic bacteriology [67,69].

### *Statistical analysis*

All statistical tests were performed using SPSS software (SPSS, Release 16.0, SPSS, Chicago, IL, USA). Two-way analysis of variance (ANOVA) and general linear model was used to evaluate the effect of time and treatments on each variable. A one-way analysis of ANOVA was done to determine the differences between different variables. Differences were considered statistically significant when $p$ <0.05, and the results were expressed as mean ± SD.

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## Author Contributions

T.M. and M.A. designed and supervised the studyand wrote and revised the manuscript draft. M.M. conceptualized the study. M.kh. conducted *in vitro* evaluations of probiotic candidates.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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